Attachment B

Amended Claims - Marked Version

1. (Reiterated) A recombinant nucleic acid encoding a CAB domain, comprising a portion of calcineurin A and a portion of calcineurin B, wherein the CAB domain forms a tripartite complex with an FKBP/CAB ligand and an FKBP domain.

2. (Amended) The recombinant nucleic acid of claim 1 wherein the calcineurin A portion of the CAB domain comprises a peptide sequence selected from any of the following peptide sequences (with reference to accession number M29550): residues 12-394 of human calcineurin A, residues 12-370 of human calcineurin A or residues 340-394 of human calcineurin A.

3. (Amended) The recombinant nucleic acid of claim 1 wherein the calcineurin B portion of the CAB domain comprises residues 3-170 of human calcineurin B (with reference to accession number M30773).

4. (Reiterated) The recombinant nucleic acid of claim 1, 2 or 3 comprising a nucleic acid sequence encoding a calcineurin A and/or calcineurin B peptide sequence which differs from a naturally occurring calcineurin peptide sequence by up to ten amino acid substitutions, deletions or insertions.

5. (Reiterated) A recombinant nucleic acid encoding a fusion protein comprising at least one CAB domain of claim 1 and at least one additional domain that is heterologous thereto.

6 (Reiterated) The recombinant nucleic acid of claim 5 wherein the heterologous domain is selected from the group comprising a DNA binding domain, a transcription regulatory domain, a cellular localizing domain and a signaling domain.

7. (Reiterated) The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from a lexA, GAL4 or composite DNA binding domain.

8. (Reiterated) The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from a p65, VP16 or AP domain.

9. (Reiterated) The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from a KRAB domain or a ssn-6/TUP-1 domain.

10. (Reiterated) The recombinant nucleic acid of claim 6 wherein the heterologous domain is or is derived from an intracellular domain of a cell surface receptor.

11. (Reiterated) A recombinant nucleic acid encoding a fusion protein containing one or more CAB domains which form a tripartite complex with an FKBP domain-containing protein and a non naturally occurring FKBP/CAB ligand preferentially over FK506.

12. (Amended) A nucleic acid composition, comprising a first recombinant nucleic acid of any of claims 5-11 [~~and~~] further comprising a second recombinant nucleic acid encoding a fusion protein comprising at least one FKBP domain and at least one additional domain that is heterologous thereto.

13. (Reiterated) A nucleic acid composition of claim 12 wherein the second nucleic acid encodes a fusion protein containing a heterologous domain that is the same or different from the heterologous domain on the first fusion protein.

14. (Reiterated) The nucleic acid composition of claim 13 wherein the first fusion protein comprises a CAB domain and a transcription activation domain and the second fusion protein comprises an FKBP domain and a DNA binding domain.

15. (Reiterated) The nucleic acid composition of claim 13 wherein the first fusion protein comprises a CAB domain and a DNA binding domain and the second fusion protein comprises an FKBP domain and a transcription activation domain.

16. (Reiterated) A nucleic acid composition of claim 12 wherein the first and second fusion proteins form a ligand dependent complex in the presence of ligand, and wherein the complex initiates a detectable biological signal.

17. (Reiterated) The nucleic acid composition of claim 16 wherein the biological signal is selected from the group comprising transcription, cell proliferation, cell differentiation, apoptosis.

18. (Reiterated) The nucleic acid composition of claim 12 wherein the composition further comprises a target gene construct.

19. (Withdrawn)

20. (Reiterated) A vector comprising a recombinant nucleic acid of any of claims 1-3 or 5-11.

21. (Reiterated) A vector comprising a recombinant nucleic acid of claim 4.

22. (Reiterated) A vector comprising a nucleic acid composition of claim 12.

23. (Reiterated) The vector of claim 20 wherein the vector is a viral vector.

24. (Reiterated) The vector of claim 22 wherein the vector is a viral vector.

25. (Reiterated) The vector of claim 23 or 24 wherein the viral vector is selected from the group consisting of adenovirus, AAV, herpesvirus, retrovirus, hybrid adenovirus/AAV, poxvirus, lentivirus.

26. (Reiterated) A host cell comprising a recombinant nucleic acid of any of claims 1-3 or 5-11.

27. (Reiterated) A host cell comprising a nucleic acid composition of claim 12.

28. (Amended) A host cell [~~cell~~] of claim 26 which is an isolated cell of human origin.

29. (Amended) A host cell [~~cell~~] of claim 27 which is an isolated cell of human origin.

30. (Reiterated) A host cell of claim 26 which is encapsulated within a biocompatible material.

31. (Reiterated) A host cell of claim 27 which is encapsulated within a biocompatible material.

32. (Reiterated) A non-human animal containing host cells of claim 26.

33. (Reiterated) A non-human animal containing host cells of claim 27

34. (Reiterated) A method for producing genetically engineered host cells comprising introducing into the cells a recombinant nucleic acid of any of claims 1-3 or 5-11 under conditions permitting DNA uptake by cells.

35. (Amended) A method for producing genetically engineered host cells comprising introducing into the cells the nucleic acid compositions [~~of any~~] of claim[~~s~~] 12[~~-18~~] under conditions permitting DNA uptake by cells.

51. (New) A method for producing genetically engineered host cells comprising introducing into the cells the nucleic acid compositions of any of claims 13 - 18 under conditions permitting DNA uptake by cells.

36. (Reiterated) The method of claim 34 wherein the nucleic acids are introduced ex vivo.

37. (Reiterated) The method of claim 35 wherein the nucleic acids are introduced ex vivo.

38. (Reiterated) The method of claim 34 wherein the cells are present within an organism.

39. (Reiterated) The method of claim 35 wherein the cells are present within an organism.

40. - 50. (Withdrawn)

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Attachment

Comments on the Delivery of Genes Using rAAV

Protocols, guidance and the benefit of working examples in the field for the design, production and use of rAAV for the delivery of genes to muscle were available to the practitioner as of applicant's priority date. The applicant's position is amply supported by disclosures made in patents, scientific literature, reviews, National Institute of Health directives and press releases from that time. Applicants provide copies of these documents in a supplemental Information Disclosure Statement submitted concurrently herewith and discuss them below.

• *(A) Prior Disclosures in the patent literature*

By the time of applicant's priority date, numerous third party patent applications were already pending, and in some cases had published, which disclosed the design, production and use of rAAV compositions for gene delivery. In particular, seven such patent applications disclosed gene delivery using AAV to various muscle cells or tissues prior to Applicant's priority date. Five of those later issued as US patents.

For instance, US Patent No. 5,658,785, from Children's Hospital in Columbus, OH, filed June 6, 1994 and issued August 19, 1997, disclosed the delivery of recombinant AAV to muscle (and other tissues):

> "Immunization of a human host with a **rAAV** of the invention involved administration by inoculation of an immunity-inducing dose of the virus by the parenteral route (e.g. by intravenous, **intramuscular** or subcutaneous injection), by surface scarification or by inoculation into a body cavity." [see, e.g., col. 5, lines 17 et seq][emphasis added here and in subsequent quotations]

The document further disclosed numerous working examples (Examples 1 - 6, pages 6 - 10) detailing rAAV vector construction, generation of stable packaging cell lines, transduction of human cell lines and successful expression of a heterologous gene product.

US Patent No. 5,658,565, filed June 24, 1994, discloses and claims a method of treating vascular occlusions, in vivo, by viral (including rAAV) delivery to endothelial cells and vascular smooth muscle cells of a gene for inducible nitric oxide synthase (iNOS) (see, e.g., col. 6, lines 40 – 54; col. 7 lines 51 – 59; col 19, lines 1 – 18; and Claims 1, 4 and 18).

US Patent No. 6,103,226, filed in 08/12/94 by Arch Development Corporation, claims a process of increasing the production of L-Dopa in a human by grafting cells, including myoblasts (col 5, lines 45 et seq), which had been ex vivo modified by viral vectors, including AAV vectors (see, e.g., col 5, lines 51 – 62 and Claims 4, 7, 10, 13, 19 and 24).

US Patent No. 5,962,424 (Arch Development Corporation), filed February 21, 1995, also claims and disclosed the use of AAV (and other viral systems) for gene delivery (see, e.g., claim 24)

WO 95/06744 (Viagene, Inc.), published March of 1994, disclosed the delivery of genes to tissue cells of an animal, either (a) in vivo or (b) ex vivo followed by introduction of the

engineered cells into the animal. Viral delivery systems disclosed in that document include AAV (and other systems) (see, e.g., page 2, lines 25 – 35; page 16, line 25 et seq.; and various other locations). Also provided is guidance on viral dosing, formulation and various modes of delivery, including intramuscular delivery (see, e.g., page 17).

WO 95/13374 (Baylor) published May 18, 1995 disclosed the use of rAAV (as well as other viral systems) to deliver a gene for the VLDL receptor in a method of treating cardiovascular disease. (see e.g., page 12, lines 18 – 28; p. 21, lines 9 – 12; and page 30, lines 4 – 6).

WO 95/13376 (Amgen and the University of Southern California), published May 18, 1995, summarized the state of the art by noting:

> "Gene transfer procedures are known to those skilled in the art and include cell transformation using calcium phosphate coprecipitation, lipofection of the target cells with liposome/gene or lipid/gene conjugates, plasmid-mediated transfer, DNA protein complex-mediated transfer and viral vector-mediated transfer. Viral vector transfer can include suitable techniques such as transfer by recombinant retroviral vectors, adenovirus vectors and **adeno-associated virus vectors**." (page 8, lines 7 et seq)

That document further disclosed

> "Cells or cell populations can be treated in accordance with the present invention either ***in vivo*** or *in vitro*. For example, in ***in vivo*** **treatments, recombinant erythropoietin vectors can be administered to the patient**, preferably in a biologically compatible solution or pharmaceutically acceptable delivery vehicle." (page 9, lines 14 et seq)

Finally, that document disclosed muscle as the preferred target tissue for in vivo gene delivery in the following passage:

> "The cells targeted for gene transfer in accordance with the present invention include any cells to which delivery of the erythropoietin gene is desired. While a variety of cells may be transfected, it was determined that **muscle cells are especially appropriate targets for gene transfer** and the expression of physiologically active amounts of erythropoietin." (page 8, lines 30 et seq)

US Patent No. 5,797,870 (Indiana University), filed June 6, 1995, discloses the use of rAAV (and other viral gene delivery systems)(see e.g., col. 5, lines 13 – 18) in a method for effecting gene therapy "selectively, efficiently and over a wide area of the heart via the pericardial space":

> "Once access to the pericardial space has been gained, gene therapy agents may be introduced to treat the heart muscle without systemic dilution or unwanted effect on other organs of the body." (Col 4, line 66 – col 5, line 18)

Those patent documents support applicant's position that rAAV was already a known tool for the in vivo delivery of genes, including the delivery of genes to various muscle cells and tissues. A collection of patent documents of interest is tabulated below. At the very least, the first seven entries from that table evidence widespread acceptance in the field of AAV-based gene delivery to muscle as of applicant's priority date

Patent /Pub Number	Priority Date	Issue/Pub Date	Assignee	Title	AAV Route
US 5,658,785	**06/06/94**	08/19/97	Children's Hospital, Inc	AAV materials and methods.	**intramuscular**
US 5,658,565	**06/24/94**	08/19/97	University of Pittsburgh	Inducible nitric oxide synthase gene for treatment of disease	Vascular smooth muscle
US 6,103,226	**08/12/94**	08/15/00	Arch Development Corporation	Genetically engineered cells that produce L. Dopa	**myoblasts**
US 5,962,424	**02/21/95**	10/05/99	Arch Development Corporation	Methods and compositions for targeting selectins	**intramuscular**
WO 95/06744	09/02/94	**03/09/95**	Viagene, Inc.	Methods of suppressing immune response by gene therapy.	**intramuscular**
WO 95/13374	11/08/94	**05/18/95**	Baylor College of Medicine	Human and mouse very low-density lipoprotein receptors and methods for use of such receptors.	**intramuscular**
WO 95/13376	10/07/94	**05/18/95**	Amgen & Univ. of So. California	Gene therapy vector for the treatment of low or defective red blood cell production.	**intramuscular**
US 5,797,870	**06/07/95**	08/25/98	Indiana University	Pericardial delivery of therapeutic and diagnostic agents	**cardiac muscle**
US 6,162,796	**09/27/95**	12/19/00	Rockefeller University	Method for transferring genes to the heart using AAV vectors	**cardiac** or vascular cells

•*(B) Prior disclosures in the scientific literature*

The scientific literature also provides relevant examples of *in vivo* gene delivery in general and gene therapy directed to muscle in several cases.

A January 8, 1995 Meeting Abstract in Modern Pathology (ref #1 in chart below) disclosed: "Cardiac gene therapy with adeno-associated virus as a means of achieving graft-specific immuno-suppression." Cardiac explants transduced with rAAV-LacZ in culture exhibited significant reporter gene expression two days after transduction.

A November 1995 meeting abstract by Podsakoff et al., published in the journal **Blood**, (ref #3 in chart below) describes heterologous gene delivery by intramuscular injection of rAAV and subsequent expression of the transgene for up to 2 months, without histopathologic evidence of an immune response.

Moreover, two 1996 publications (#2 and #5, below) report long term gene expression following earlier gene delivery by intramuscular administration of recombinant AAV. All of these studies were calculated to have been initiated in 1995, based upon the reported

duration of gene expression achieved. The Nov 1996 **J Virology paper** (ref #2 in chart below), disclosed that a single administration of a rAAV vector was capable of reporter gene expression which "persisted for more than 1.5 years". Based upon the acceptance date of the article (July 1996) one can calculate a date for delivery of the rAAV in January or February of 1995, as shown for other publications in the table below. The PNAS paper, ref #5, below, disclosed that AAV-directed expression in fore limb muscle extended for up to 40 weeks, from which one calculates a rAAV administration date no later than September/October of 1995, if not earlier. See also refs #4 and #6 (AAV to scalp muscle and rat heart, respectively)

Again, these documents evidence the availability and the acceptance in the art of technology for the design, production and use of rAAV materials and methods for the delivery of genes to muscle.

Citation	Tissue Transduced	Length of Expression	Submission Date	Deduced Start Date of Experiment
#1 Jan 8, 1995 Modern Pathology, Vol. 8(1): 33A Meeting Abstract #178	Cardiac explants	----	unknown	No later than January 1995
#2 Nov 1996 J Virology, 70(11): 8098-108	Hind leg muscle	Over 1.5 years	July 31 1996	January/February 1995
#3 Nov 15, 1995 Blood, Vol. 83(10), Suppl 10 Pg. 1004a, Abstract #4004	Hind leg muscle	2 months	unknown	August/September 1995
#4 May 1, 1996 Human Gene Therapy, Vol. 7: 841-850	Striated scalp muscle	---	October 16. 1995	September 1995
#5 Nov 1996, PNAS, Vol. 93: 14082-14087	Tibialis muscle	40 weeks	July 5, 1996	September/October 1995
#6 Dec 1996, Ann Thoracic Surg: 62(6): 1669-76	Heart muscle	6 months	unknown	May 1996

Moreover, several scientific publications demonstrate the *in vivo* use of AAV in tissues other than muscle prior to the Applicant's priority date. In 1993 Flotte disclosed rAAV delivery of a heterologous gene in the respiratory epithelium of rabbits (PNAS 1993, 90: 10613-10617). During and Leone review the successful *in vivo* neuronal expression of transgenes delivered by rAAV, reported before our 1st priority date (During & Leone, 1996, 3: 292-300).

• *C Scientific Reviews*

AAV had become well enough characterized and recognized as a preferred vector for use in gene delivery that it became the subject of numerous reviews, in some cases dating back prior to applicant's priority date. These reviews provided interesting background information on AAV biology, a wealth of helpful guidance and practical information on the production and use of rAAV and an overview of the state of the art as viewed prior to and just subsequent to applicant's priority date. Below is a chart of several such reviews.

Authors	Title	Citation
Ali et al.	The use of DNA viruses as vectors for gene therapy.	1994 Gene Therapy, 1: 367-84.
Srivastava	Parvovirus-based vectors for human gene therapy.	1994 Blood Cells., 20(2-3): 531-6;
Kremer & Perricaudet	Adenovirus and adeno-associated virus mediated gene transfer.	1995 Br Med Bull. 51: 31-44.
Flotte & Carter	Adeno-associated virus vectors for gene therapy	1995 Gene Therapy, 2: 357-62
During & Leone	Adeno-associated virus vectors for gene therapy of neurodegenerative disorders.	1995-96 Clin Neurosci., 3: 292-300 (1996)
McKeon & Samulski	Meeting Report: NIDDK Workshop on AAV Vectors: Gene transfer into Quiescent cells	1996 Human Gene Therapy, 7: 1615-1619
Carter	The promise of adeno-associated virus vectors.	1996 Nature Biotechnology, 14; 1725-1726

Notes on those reviews are presented below in the same order as the reviews are listed in the foregoing chart.

Ali et al. disclosed the production of high titer AAV stocks and the value of AAV vectors in the following:

> "A number of features make AAV an attractive system for the use as a gene therapy vector. AAV has not been implicated in any human disease and integration of the AAV genome does not appear to have any effect on cell replication. Furthermore, AAV can be concentrated by centrifugation in excess of 10^{12} infectious units per milliliter and are heat stable permitting inactivation of helper virus (adenovirus) by subjecting preparations to 60^{O} C for 30 min." (See p. 378, beginning at bottom left column)

Srivastava disclosed that high efficiency transduction was attainable with AAV:

> "Using the recombinant AAV vector system, it is feasible to obtain high-efficiency transduction of slow- or non-cycling primary hematopoietic stem and progenitor cells, without the need for prestimulation with cytokines, which could potentially lead to differentiation of these cells before transplantation." (see Abstract)

Flotte & Carter disclosed that AAV vectors delivery systems had become advanced enough to enter clinical trials, noting:

> "Adeno-associated virus type 2 (AAV) is a non-pathogenic DNA virus which has been utilized as a eukaryotic gene transfer vector in vitro and in vivo. AAV possesses a unique set of characteristics, which may make it useful for human gene therapy. AAV infection does not require host cell proliferation, although expression from AAV vectors may exhibit a relative preference for actively dividing cells. Both wild type AAV and AAV vectors tend to persist in infected cells for prolonged periods of time, without any significant adverse consequences for the host." (See abstract)

During & Leone disclosed the following:

"Adeno-associated virus (AAV) shows significant potential as a gene delivery system." The authors go on to state that the "characteristics [of AAV] make it particularly attractive as a gene delivery vehicle. As the enthusiasm driving the proliferation of clinical gene transfer protocols [with non-AAV delivery approaches] has dampened recently due to the lack of clinical success often reflecting immunogenicity and inefficiency of the gene transfer methods used in these trials, AAV with minimal (if any) toxicity and high efficiency in a wide range of cells and tissues, may become the vector-of-choice for many applications." (See Abstract)

McKeon and Samulski summarized a NIH sponsored forum on AAV vectors held in December of 1995 and recent work on AAV vectors. Among other things, they disclosed that :

> *"In vivo* experiments with rAAV suggest that some cell types in the brain and muscle are naturally permissive for AAV, and may not need an agent to stimulate transduction. In mice, where muscle was transduced with rAAV, transgene persistence and gene expression was observed for over a year and appeared to remain constant with time." (See p. 1617, left column, 3d paragraph)

The authors report that AAV production could be facilitated by a chapter in a laboratory manual for researchers dedicated to AAV protocols, which they cite. The fact that rAAV technology had become commonplace enough by December 1995 to have already become the subject of a lab manual lends considerable credence to Applicant's assertion that the rAAV teachings in the art were abundant and enabling of the present invention.

Carter, commenting on prior rAAV experiments, albeit in a subsequent review, disclosed the following: "The primary objectives of both [AAV clinical] trials were to assess safety, and to provide data on vector delivery and persistence in airway cells with repeat delivery. **These goals were met**." The document further discloses: "More recent studies, mostly in rodents, have shown prolonged expression of reporter genes following delivery of AAV vectors to the liver, brain, eye, and skeletal muscle. In skeletal muscle, expression extended for as long as 18 months." (See page 1726)

•(D) Documents from the National Institutes of Health

National Heart Lung & Blood Institute of the NIH, in its Fiscal Year Report for 1994 noted: "The second approach [in treating hemophilia] used recombinant **adeno-associated virus vectors, administered by intramuscular injection for muscle tissue expression of hfIX [human Factor IX]**. The reports showed hfIX expression in immunodeficient mice for at least 6-10 months. These studies represent an important advance in gene therapy research for hemophilia B. " (This document, "NHLBI AIDS related research, FY 1994" was found at the following URL: http://www.nhlbi.nih.gov/resources.aids/fy94.htm).

In a 1996 NIH Guide, AAV vectors were reported to have already become the leading choice of gene delivery systems in 1995, as seen in the following statements. " On December 6, 1995, the NIDDK conducted a Workshop entitled, "AAV Vectors: Gene Transfer into Quiescent Cells." The Workshop explored the potential applications for one novel vector system, AAV, which exhibits many desirable characteristics for

treatment of metabolic diseases." (See page 1, bottom paragraph). The Guide implied AAV systems had reached a degree of maturity as a biological tool: "Although this Workshop was focused on AAV vectors, it served as a prototype for the problems encountered by investigators developing any new vector system." (See page 2, 1st paragraph)(NIH GUIDE, Volume 25, Number 35, October 18, 1996).